

Holliday junction recognition protein as a prognostic biomarker and therapeutic target for oral cancer

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Abstract. Since oral cancer (OC) is highly malignant and the efficacy of standard treatments is limited, the development of new therapeutics is urgently awaited. To identify potential molecular targets for new OC diagnosis and therapies, we screened oncoantigens by gene expression profile and focused on Holliday junction recognition protein (HJURP), a mammalian centromere-specific chaperone. HJURP was found to be highly expressed in the majority of OC cell lines and tissues as compared to normal oral epithelial cells. Tissue microarray analysis confirmed that HJURP was expressed in 103 (67.8%) of 152 OC tissue specimens, but expression in normal oral tissues was limited. Positive HJURP expression was significantly correlated with shorter overall survival ($P=0.003$). Depletion of HJURP by small-interfering RNAs dramatically inhibited the growth of OC cells by inhibition of cell cycle progression and induced senescence of OC cells. In addition, inhibition of the interaction between HJURP and CENP-A significantly suppressed the growth of OC cells. These results indicate that HJURP is a potential prognostic biomarker, and targeting HJURP and its molecular pathway presents a new strategy for the development of treatments against OC.

Introduction

Oral cancer (OC) is highly malignant, and patient prognosis remains poor even after standard treatments. OC is a leading cause of cancer-related death, and a total of 354,864 new cases of OC were diagnosed worldwide in 2018 (1,2). The high prevalence of OC has been associated with the use of tobacco, including smokeless tobacco, and heavy alcohol consumption, particularly in developing countries in Asia (2-4). Oral squamous cell carcinoma accounts for approximately 90% of all OC cases (5). Either surgery or radiotherapy is recommended as standard therapies for early stage OC. In highly advanced stages, concurrent systemic chemotherapy with radiotherapy is suggested with surgical removal of residual tumors if feasible. During the past few decades, the 5-year overall survival of OC has remained unfavorable due to the low sensitivity to therapies and metastatic potential that are thought to be caused by genetic aberrations. The 5-year survival rate of OC is up to 80% in the early stage of disease and only 20-30% in advanced stages, which is dependent on various factors, including the primary site in the oral cavity, comorbidity, and selection of treatment (6,7). Overall survival of OC patients has improved by 15% over the last 50 years, but only 5% over the last 20 years (8). Recent developments in new treatments, including molecular-targeted therapy and immunotherapy, have improved the survival of OC patients. Cetuximab, an inhibitor of epidermal growth factor receptor (EGFR), is reportedly efficacious against advanced squamous cell carcinoma of the head and neck (9). Treatment with nivolumab, an anti-programmed cell death-1 (PD-1) antibody (Ab), following cetuximab has improved the overall survival of patients with advanced head and neck cancers (10). Nonetheless, the efficacy of these treatments against OC remains limited. Therefore, more effective molecular therapies targeting cancer-specific molecules with less adverse events in combination with personalized medicine using several types of cancer biomarkers are urgently needed.

Gene expression profile analysis and subsequent tissue microarray analysis of a variety of solid tumor tissues have been employed to identify potential molecular targets for cancer diagnostics and therapeutics. Dozens of oncoantigens, which are essential for disease progression of various solid

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Abbreviations: DAPI, 4',6-diamidino-2-phenylindole; HJURP, Holliday junction recognition protein; FBS, fetal bovine serum; HOMKs, human oral mucosal keratinocytes; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; OC, oral cancer; p21/CDKN1A, cyclin-dependent kinase inhibitor 1A; SA- β -Gal, senescence-associated β -galactosidase; SAHF, senescence-associated heterochromatic foci

Key words: Holliday junction recognition protein, oral cancer, biomarker, therapeutic target, oncoantigen

cancers, in addition to various molecules involved in cell cycle progression and/or cell survival have been identified (11-37). As a potential biomarker and molecular target for the treatment of OC, Holliday junction recognition protein (HJURP) is highly expressed in the majority of OCs, while expression is comparatively low in normal tissues. As reported in our previous study, HJURP contributes to the immortality of cancer cells through the DNA double-strand break repair pathway by interacting with MSH5 and NBS1 (24). HJURP is a part of a centromeric protein with four domains that is required for centromere protein A (CENP-A) nucleosome assembly at the centromeres to ensure accurate chromosomal segregation during cell division (38,39). High expression of HJURP was found to be associated with a poorer clinical outcome of patients with non-small cell lung cancer (24), hepatocellular carcinoma (40), gliomas (41), and ovarian cancer (42), and was proposed as a predictive marker of responses to radiotherapy of breast cancer patients (43). However, the precise role in OC and the clinical potential of HJURP as a molecular target remain unclear. Therefore, the aim of the present study was to investigate the role of HJURP in the malignant nature of OC and its potential as a diagnostic and prognostic tissue biomarker and a therapeutic target for OC.

Materials and methods

Cell lines and clinical tissue samples. Human OC cells (CAL 27, Ca9-22, FaDu, HSC2, HSC3, HSC4, and SCC-9) were cultured in medium supplemented with 10% fetal bovine serum (FBS; Gibco, Thermo Fisher Scientific Inc.) and 1% penicillin/streptomycin (Wako Pure Chemical Industries) at 37°C under a humidified atmosphere of 5% CO₂/95% air. Human primary oral mucosal keratinocytes (HOMKs) were commercially purchased and cultured in medium supplemented with EpiLife defined growth supplement (Gibco, Thermo Fisher Scientific Inc.). The features of all cells are summarized in Table I. The requirement for ethics approval for the use of commercially available primary human cells such as HOMK cells in this study was waived by the ethics committee. For real-time quantitative polymerase chain reaction (qPCR) experiments, 14 frozen oral squamous cell cancer tissues (4 female, 10 male patients; median age, 60 years; age range, 45-74 years; all cases were Caucasians) were purchased from ProteoGenex, Inc., and commercially available normal tongue tissue polyA RNA was obtained from Clontech Laboratories, Inc. Moreover, 152 existing formalin-fixed paraffin-embedded OC tissues and adjacent normal oral tissues obtained from patients (66 females and 86 males; median age, 69 years; age range, 28-92 years; all cases analyzed in this study were Asians) who underwent curative surgery with adjuvant chemotherapy or neoadjuvant chemotherapy at Kumamoto University between 2004 and 2012 were used for immunohistochemical analysis on tissue microarrays. The Union for International Cancer Control TNM classification was used to determine the clinical stage of the OC samples. The study protocol and the use of existing clinical materials in this study were approved by the relevant Ethics Committees [Kumamoto University; Shiga University of Medical Science (no. G2009-163)] based on the national ethical guidelines for human subjects. It was confirmed that this study was fully ethically compliant and

the informed consent was waived due to the retrospective nature of the study and in accordance with the national ethical guidelines.

qPCR. Total RNA was isolated from cultured cells and clinical tissues using the Maxwell® 16 LEV simplyRNA Cells Kit (Promega Corp.) in accordance with the manufacturer's protocol. For reverse transcription, cDNA was synthesized from total RNA using PrimeScript™ RT Master Mix (Takara Bio Inc.). Samples were incubated at 37°C for 15 min and 85°C for 5 sec. The qPCR experiments were performed with TaqMan Fast Universal PCR Master Mix (Thermo Fisher Scientific Inc.) and a QuantStudio™ 3 Real-Time PCR System (Applied Biosystems; Thermo Fisher Scientific, Inc.) in accordance with the manufacturers' protocols. All experiments were performed in triplicate. The primers Hs01565312_m1 and Hs01060665_g1 (Applied Biosystems; Thermo Fisher Scientific, Inc.) were used for amplification of *HJURP* and *ACTB* (as an internal control), respectively. The thermal cycling conditions consisted of an initial denaturation step at 95°C for 20 sec followed by 40 cycles at 95°C for 1 sec and 60°C for 20 sec. Comparative *HJURP* mRNA expression was calculated by the $2^{-\Delta\Delta C_q}$ method (44) with *ACTB* mRNA expression as a reference.

Western blot analysis. The cells were washed with cold phosphate-buffered saline (PBS) (-) and lysed with radioimmunoprecipitation assay buffer with a protease inhibitor cocktail (Thermo Fisher Scientific Inc.). After homogenization, the cell lysates were cooled on ice for 30 min and then centrifuged at 15,000 rpm for 15 min to separate the supernatant from cellular debris. The amount of total protein was quantified with a detergent compatible protein assay kit (Bio-Rad Laboratories, Inc.) and then mixed with sample buffer, boiled at 100°C for 5 min, and incubated at room temperature for 5 min. Proteins were separated by electrophoresis using 10% Mini-PROTEAN® TGX™ Precast Gels (Bio-Rad Laboratories, Inc.) and then transferred to Trans-Blot® Turbo™ 0.2 µm polyvinylidene fluoride membranes (Bio-Rad Laboratories, Inc.), which were blocked with Block Ace solution (DS Pharma Biomedical) and incubated overnight at 4°C with a rabbit polyclonal Abs against HJURP (dilution, 1:500; catalog no. HPA008436; Sigma-Aldrich; Merck KGaA), rabbit monoclonal Abs against p21 (1:1,000; catalog no. 2947; Cell Signaling Technology, Inc.) and Lamin B1 (1:1,000; catalog no. 13435; Cell Signaling Technology, Inc.), a mouse monoclonal Ab against Myc-Tag (1:1,000; catalog no. 2276; Cell Signaling Technology, Inc.), and a rabbit polyclonal Ab against β-actin (1:2,000; catalog no. 4970; Cell Signaling Technology, Inc.). After washing with Tris-buffered saline containing Tween-20 (Cell Signaling Technology, Inc.), the membranes were incubated with anti-rabbit (1:3,000; catalog no. NA934V; GE Healthcare) or anti-mouse (1:2,000; catalog no. NA931V; GE Healthcare) horseradish peroxidase-conjugated secondary Abs for 1 h at room temperature, and visualized using enhanced chemiluminescence reagent with a Fusion Solo S image analyzer (Vilber Lourmat).

Immunocytochemical analysis. Cultured cells were grown on Lab-Tek II chamber slides (Nalge Nunc International), washed

Table I. Human OC cell lines and HOMKs.

Cell line	Histology	Resource distributor	Catalog no.
CAL 27	Squamous cell carcinoma of tongue	ATCC	CRL-2095
Ca9-22	Gingival squamous cell carcinoma	RIKEN BRC	RCB1976
FaDu	Squamous cell carcinoma of pharynx	ATCC	HTB-43
HSC2	Squamous cell carcinoma of mouth	RIKEN BRC	RCB1945
HSC3	Squamous cell carcinoma of tongue	RIKEN BRC	RCB1975
HSC4	Squamous cell carcinoma of tongue	RIKEN BRC	RCB1902
SCC-9	Squamous cell carcinoma of tongue	ATCC	CRL-1629
HOMK	Human oral mucosa keratinocytes	Cell Research Corp. Pte Ltd	hOMK100

ATCC, American Type Culture Collection; RIKEN BRC, RIKEN BioResource Center; OC, oral cancer; HOMKs, human oral mucosal keratinocytes.

with cold PBS (-), fixed with 4% paraformaldehyde for 15 min, and permeabilized with 0.2% Triton X-100 in PBS (-) for 2 min at room temperature. Non-specific binding was blocked by 3% bovine serum albumin (BSA; Wako Pure Chemical Industries) in PBS for 30 min and then incubated with a rabbit polyclonal Abs against HJURP (1:100; HPA008436; Sigma-Aldrich; Merck KGaA) in PBS (-) supplemented with 1% BSA overnight at 4°C in a wet box. After washing with PBS (-), Alexa Fluor 488-conjugated anti-rabbit Abs (1:800; catalog no. A11008; Life Technologies, Inc.) were applied for 90 min at room temperature in the wet box with protection from light. Afterward, the cells were mounted on glass slides using VECTASHIELD® Antifade Mounting Medium with DAPI (4',6-diamidino-2-phenylindole; Vector Laboratories, Inc.) and visualized with a confocal laser scanning microscope at x63 magnification (Leica TCS SP8 X; Leica Microsystems).

Immunohistochemical analysis and tissue microarray. To verify the biological and clinicopathological significance of HJURP in clinical OC tissues, HJURP protein expression was examined using tissue microarrays. Tumor tissue microarrays were created from formalin-fixed paraffin-embedded primary OC tissues resected from 152 patients (59 who underwent curative surgery with neoadjuvant chemotherapy and 93 who underwent curative surgery with adjuvant chemotherapy). For construction of the tissue microarrays, the tissue specimens were cut into sections, which were stained with hematoxylin and eosin to identify appropriate tumor areas for sampling. Three to five tissue cores with a diameter of 0.6 mm were taken from selected areas of each tumor donor block using a tissue microarrayer (Beecher Instruments Inc.) and placed into a recipient paraffin block. A core of normal oral epithelial tissue was obtained from each specimen and 5-μm-thick sections of the tissue microarray blocks were used for immunohistochemical analysis.

Tissue microarray slides were deparaffinized in xylene and rehydrated in graded concentrations of ethanol. Then, antigen retrieval was conducted by heating the samples in a microwave oven in Target Retrieval Solution at pH 6.0 (Dako). Endogenous peroxidase was blocked using hydrogen peroxide and the slides were incubated in Protein Block Serum-Free solution (Dako) for 30 min, followed by incubation with rabbit polyclonal

Abs against HJURP (1:500; HPA008436; Sigma-Aldrich; Merck KGaA) overnight at 4°C. After washing, the slides were incubated with EnVision + System-HRP Labeled polymer anti-rabbit secondary Abs (Dako) for 30 min, followed by 3,3'-diaminobenzidine (Dako) as the substrate chromogen and hematoxylin (Dako) as a nuclear counterstain. Images of the immunostained samples were acquired with a NanoZoomer® whole slide scanner (Hamamatsu Photonics K.K.) and positivity of the HJURP protein was semi-quantitatively analyzed by three independent investigators without prior knowledge of the clinicopathological data. Staining of more than 10% of the tumor nuclei was considered positive for HJURP expression, while staining of less than 10% of the tumor nuclei was considered negative as previously described (24). A specimen was considered positive by consensus of all three investigators. Since most positive cases showed homogenous nuclear staining in tumor tissues, the cutoff value of the staining index of HJURP in positive tissues was not used in this study.

RNA interference assay. HSC4 and Ca9-22 cells (1x10⁶) were plated on 10-cm dishes and transfected with either small-interfering RNAs (siRNAs) against HJURP or control siRNAs using Lipofectamine 2000 reagent (Invitrogen; Thermo Fisher Scientific Inc.) in accordance with the manufacturer's recommendations, as previously described (24). The target sequences of the siRNAs were as follows: *si-HJURP-#1*, 5'-GUCAGU UGCUUGGGCCUU A-3'; *si-HJURP-#2*, 5'-CAAGCAUCA UCUCACCAA-3'; Control siRNA-1 (*LUC*), 5'-CGUACG CGGAUACUUCGATT-3'; and Control siRNA-2 (*EGFP*), 5'-GAAGCAGCAGACUUCUUCTT-3' (Sigma-Aldrich; Merck KGaA). Knockdown of HJURP expression by siRNAs was confirmed by western blot analysis using Abs against HJURP. It was also confirmed that si-LUC and si-EGFP are suitable controls with no off-target effect on the various types of cancer cells including OC as previously described (11-37).

Cell viability assay. HSC4 or Ca9-22 cells (1x10⁴) transfected with siRNAs against HJURP or control siRNAs were plated in the wells of a 6-well plate with growth medium containing 10% FBS (Thermo Fisher Scientific Inc.). The viability of the cells was determined on post-transfection day 7 with a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium

bromide (MTT) dye reduction assay using Cell Counting Kit-8 solution (Dojindo Laboratories).

Colony formation assay. HSC4 and Ca9-22 cells (1×10^5) transfected with siRNAs against HJURP or control siRNAs were cultured on 10-cm dishes. After 7 days of incubation, the cells were washed three times with PBS (-) and fixed with 4% paraformaldehyde phosphate-buffered solution (Wako Pure Chemical Industries) for 1 h. After drying at room temperature, the cells were stained with Giemsa staining solution (Wako Pure Chemical Industries) and images were captured using a PIXUS-MP990 multifunction device (Canon).

Flow cytometry. HSC4 and Ca9-22 cells (1×10^6) transfected with siRNAs against HJURP or control siRNAs were used for cell cycle analysis using a CycleTEST™ PLUS DNA Reagent Kit (BD Biosciences), while quantitative analysis of senescence-associated β -galactosidase (SA- β -gal)-positive cells was conducted using a Cellular Senescence Detection Kit-SPiDER- β Gal (catalog no. SG03; Dojindo Laboratories) in accordance with the manufacturers' instructions. After transfection, the cells were harvested, filtered through a 70- μ m nylon mesh, and kept on ice in the dark. The cells (1×10^4) were analyzed within 3 h using a FACSVerse flow cytometer (BD Biosciences).

Live cell imaging. HSC4 and Ca9-22 cells transfected with siRNAs against HJURP or control siRNAs were cultured on 35-mm glass dishes containing growth medium supplemented with 10% FBS. Time-lapse images were acquired every 60 min following HJURP knockdown using the EVOS FL Auto Cell Imaging System (Thermo Fisher Scientific Inc.) to reveal cellular dynamics.

SA- β -gal staining and detection of senescence-associated proteins. HSC4 or Ca9-22 cells (1×10^4) transfected with siRNAs were cultured on 35-mm culture dishes, washed with PBS (-), and stained with a Senescence β -galactosidase Staining Kit (Cell Signaling Technology). The presence of SA- β -gal activity was determined by incubating cells with X-Gal solution overnight at 37°C in a dry incubator without CO₂. Stained cells were evaluated under a light microscope. HSC4 and Ca9-22 cells (1×10^3) transfected with siRNAs were used for quantitation of SA- β -gal activity with a Cellular Senescence Plate Assay Kit-SPiDER- β Gal (catalog no. SG05; Dojindo Laboratories) and a Cell Count Normalization Kit (catalog no. C544; Dojindo Laboratories) for normalization in accordance with the manufacturer's instructions. Flow cytometric quantitative analysis of cells positive for SA- β -gal was performed using a Cellular Senescence Detection Kit-SPiDER- β Gal (catalog no. SG03; Dojindo Laboratories) as mentioned above. All assays were performed in triplicate. Expression levels of senescence-associated secretory phenotype (SASP) markers were detected using lysates of HSC4 and Ca9-22 cells with a human cytokine Ab array according to the manufacturer's protocol (Human Angiogenesis Antibody Array; catalog no. ab193655; Abcam).

Dominant-negative peptide assay. Short amino acid sequences derived from the TLTY box of the CENP-A binding

domain of HJURP (TLTYETPQ 54-61; ref. 45), which is a direct binding site for CENP-A, were linked to a membrane transducing 11 poly-arginine sequence (11R; ref. 32). The cell permeable peptide 11R-HJURP^{TLTY54-61} (RRRRRRRRRR RR-GGG-TLTYETPQ) and a scramble peptide (RRRRRR RRRRRR-GGG-PTQTYLET) as a control (Sigma-Aldrich) with >95% purity were synthesized. HSC4 and Ca9-22 cells (1×10^4) were incubated with the peptides at different concentrations (20-200 μ M) for 3 days to examine the growth suppressive effects of a cell permeable peptide in OC cells. The viability of treated cells was evaluated using the MTT assay.

To confirm the effect of the synthesized peptides on the interaction between HJURP and CENP-A in OC cells, immunoprecipitation experiments were performed using OC cells treated with or without synthesized peptides and polyclonal Abs against HJURP (Sigma-Aldrich; Merck KGaA) for immunoprecipitation and mouse monoclonal Abs against CENP-A (1:1,000; catalog no. MA1-20832; Invitrogen; Thermo Fisher Scientific Inc.) for western blot analysis. For the immunoprecipitation experiments, protein samples were incubated with polyclonal Abs against HJURP (1:100; HPA008436; Sigma-Aldrich; Merck KGaA) overnight at 4°C. After 2 h of incubation with 20 μ l of Pierce Protein A/G Plus Agarose beads (catalog no. 20423, Thermo Fisher Scientific Inc.), proteins bound to the beads were collected by centrifugation at 3,000 rpm for 1 min at 4°C and washed with lysis buffer. Then, the beads were resuspended in Laemmli sample buffer and boiled for 5 min for western blot analysis, which was performed using monoclonal primary Abs (Invitrogen; Thermo Fisher Scientific Inc.) and anti-mouse secondary Abs (1:2,000; GE Healthcare) against CENP-A.

Statistical analysis. All statistical analyses were conducted using StatView 5.0 statistical software (SAS Institute, Inc.) and IBM SPSS Statistics for Windows, version 25.0. (IBM Corp.). Differences between groups of cell-based assays were compared using the Student's t-test (unpaired data), and multiple comparisons were conducted with one-way ANOVA followed by Tukey's post hoc test. Fisher's exact test was used to assess the correlation of HJURP expression levels determined by tissue microarray analysis with relevant clinicopathological variables, such as patient age, sex, primary tumor region, pT (pathologic tumor) classification, and pN (pathologic lymph node) classification. Tumor-specific survival curves were drawn from the date of surgery to the time of death related to OC or the last follow-up observation. Kaplan-Meier curves were calculated for each relevant variable and HJURP expression. Differences in survival times among patient subgroups were analyzed using the log-rank test. Univariate and multivariate analyses were performed with Cox proportional hazards models to identify prognostic factors of OC patients. First, associations between death and possible prognostic factors, including positive HJURP expression, age, sex, primary region, pT classification, pN classification, and treatment (curative surgery with adjuvant chemotherapy vs. curative surgery with neoadjuvant chemotherapy) were analyzed. Second, multivariate Cox analysis was applied in stepwise procedures that always forced positive HJURP expression

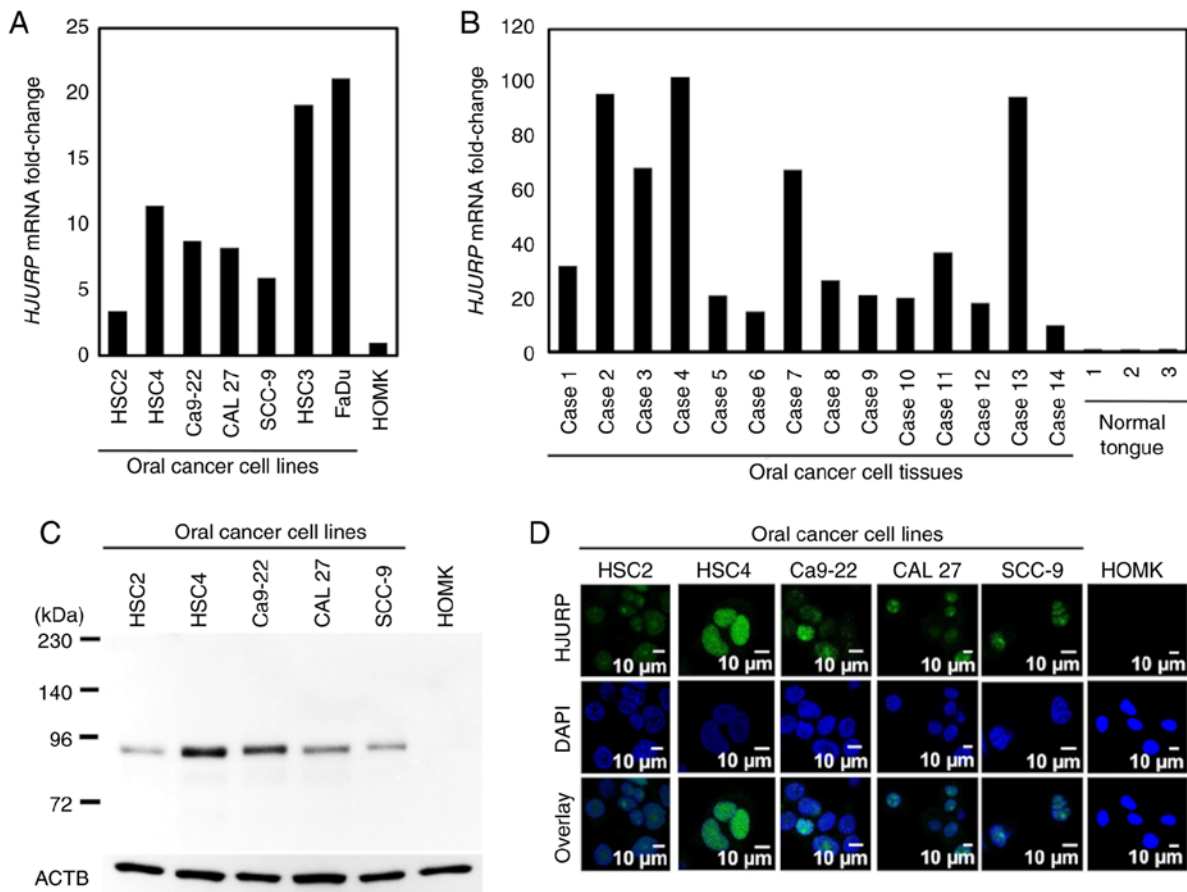


Figure 1. HJURP expression in OC cells and tissues. (A and B) Detection of *HJURP* transcripts by qPCR in OC cell lines and tissues. (C) HJURP protein expression detected by western blot analysis in OC cell lines. (D) Subcellular localization of HJURP protein in OC cell lines and HOMKs. The cells were stained with a rabbit polyclonal Ab against HJURP (green) and DAPI (blue). HJURP, Holliday junction recognition protein; HOMKs, human oral mucosal keratinocytes; OC, oral cancer.

into the model along with each significant variable. As significant prognostic factors were continually added to the model, independent factors with a P-value <0.05 were considered statistically significant.

Database analysis. The relationship between *HJURP* expression and survival of head and neck cancer patients was evaluated with reference to the ProgGene database (<http://genomics.jefferson.edu/proggene/>) and GEPIA (Gene Expression Profiling Interactive Analysis) database (<http://gepia2.cancer-pku.cn/#index>). Signaling pathways related to HJURP were screened against the ONCOMINE database (<https://www.oncomine.org/resource/login.html>) and GSEA (Gene Set Enrichment Analysis) database (<https://www.gsea-msigdb.org/gsea/msigdb/search.jsp>).

Transient expression of *HJURP*. Myc-DDK-tagged *HJURP*-expression plasmids (catalog no. RC201283) and its control pCMV6-Entry plasmids (catalog no. PS100001) were purchased from OriGene Technologies, Inc. HSC2 and SCC9 cells were transfected with these vectors using FuGENE transfection reagent (Promega Corp.) according to the manufacturer's instructions. Cell viability was measured by MTT assay using Cell Counting Kit-8 solution (Dojindo Laboratories) and colony formation assay with Giemsa staining (Wako Pure Chemical Industries).

Results

Expression of *HJURP* in OC cell lines and tissues. qPCR revealed *HJURP* mRNA expression in all OC cell lines and tissues, but very low expression in the HOMKs and normal tongue tissues (Fig. 1A and B). Western blot analysis showed high HJURP protein expression in all OC cell lines as compared with that observed in the HOMKs (Fig. 1C). Immunocytochemical staining showed that HJURP protein was mainly localized in the nucleus of cancer cells, but expression was limited in the HOMKs (Fig. 1D).

HJURP is associated with the poor prognosis of OC patients.

To develop new anticancer drugs with minimum risk of adverse effects and highly cancer specific biomarkers, we validated HJURP as a potential therapeutic target that is frequently overexpressed in OC cells, but scarcely expressed in normal vital organs. HJURP expression was examined by immuno-histochemical analysis in normal tissues (heart, lung, kidney, liver, and tongue as representative vital organs; placenta, and testis as references) and OC tissues. The HJURP protein was mainly observed in the nuclei of OC cells, with weak expression in testis cells and limited detection in the remaining normal tissues (Fig. 2A). Furthermore, tissue microarrays of the tissue samples from OC patients who underwent curative surgery confirmed HJURP protein expression in 103 (67.8%)

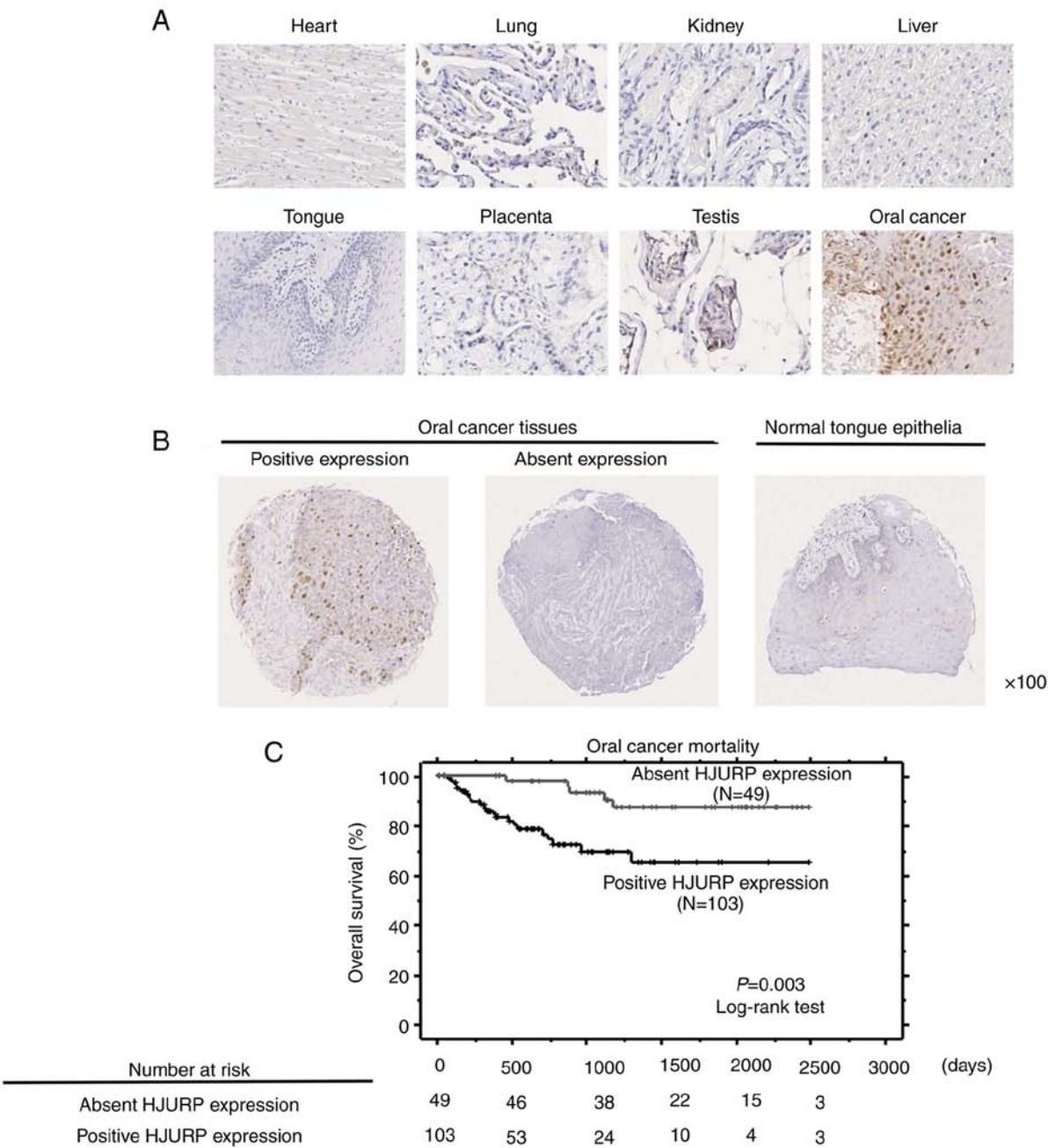


Figure 2. HJURP expression is associated with poor prognosis of OC patients. (A) Immunohistochemical staining of HJURP protein in normal human and OC tissues. (B) Expression pattern of HJURP protein in representative OC tissues and healthy tongue tissues (original magnification, x100). (C) Kaplan-Meier analysis of the survival of OC patients based on HJURP positivity ($P=0.003$; by log-rank test). HJURP, Holliday junction recognition protein; OC, oral cancer.

of 152 OC specimens, but not in the normal tongue epithelial tissues (Fig. 2B). Evaluation of the association of HJURP expression with clinicopathological parameters showed that HJURP expression was significantly correlated to age (higher in patients ≥ 65 years, $P=0.0216$ by Fisher's exact test; Table II). The results of Kaplan-Meier analysis revealed that HJURP protein expression was significantly correlated with a poorer prognosis of OC patients ($P=0.003$, by log-rank test; Fig. 2C). Univariate analysis was conducted to investigate the correlation between possible prognostic factors, including HJURP expression status (absent vs. positive), age (<65 vs. ≥ 65 years), sex (female vs. male), tumor region (tongue vs. other regions), pT classification (T1-2 vs. T3-4), pN classification (N0 vs. N1-2), and treatment (curative surgery with adjuvant chemotherapy

vs. curative surgery with neoadjuvant chemotherapy). The results showed that positive HJURP expression and advanced pN stage (N1-2) were significantly associated with a poorer prognosis of the OC patients ($P=0.0057$ and 0.0014 , respectively, Table III). Furthermore, multivariate analysis revealed that positive HJURP expression and advanced pN stage were independent prognostic factors ($P=0.0093$ and 0.0031 , respectively, Table III).

To validate the potential of HJURP as a prognostic biomarker, the prognostic value of *HJURP* gene expression was investigated using the ProgGene database. The results showed that *HJURP* expression was significantly associated with poor prognosis of patients with head and neck cancers, including OC (dataset no. E-MTAB-1328; $P=0.0286$). Furthermore,

Table II. Association of HJURP protein expression in OC tissues with patient characteristics.

Characteristics	Total no. of patients (N=152)	Positive HJURP expression (N=103)	Absent HJURP expression (N=49)	P-value (positive vs. absent)
Sex				
Male	86	61	25	0.3835
Female	66	42	24	
Age (years)				
<65	60	34	26	0.0216 ^a
≥65	92	69	23	
Region				
Tongue	76	49	27	0.4878
Others ^b	76	54	22	
pT classification				
T1-2	93	61	32	0.5935
T3-4	59	42	17	
pN classification				
N0	109	69	40	0.0824
N1-2	43	34	9	

^aP<0.05 (Fisher's exact test). ^bGingiva, buccal mucosa and others. OC, oral cancer; HJURP, Holliday junction recognition protein.

Table III. Cox's proportional hazards model analysis of prognostic factors in patients with OC.

Variables	HR	95% CI	Unfavorable/Favorable	P-value
Univariate analysis				
HJURP expression	3.983	1.495-10.609	Positive/Absent	0.0057 ^a
Age (years)	2.051	0.901-4.668	≥65/<65	0.0869
Sex	2.090	0.988-4.422	Female/Male	0.0539
Region	1.658	0.782-3.517	Others ^b /Tongue	0.1872
T-factor	1.610	0.766-3.388	T3-4/T1-2	0.2091
N-factor	3.345	1.592-7.028	N1-2/N0	0.0014 ^a
Treatment	1.368	0.647-2.894	Neoadjuvant/Adjuvant chemotherapy	0.412
Multivariate analysis				
HJURP expression	3.704	1.381-9.933	Positive/Absent	0.0093 ^a
N-factor	3.083	1.463-6.498	N1-2/N0	0.0031 ^a

^aP<0.05. ^bGingiva, Buccal mucosa, others. OC, oral cancer; HR, hazard ratio; CI, confidence interval; HJURP, Holliday junction recognition protein.

GEPIA for analysis of the RNA sequencing data from The Cancer Genome Atlas and the Genotype-Tissue Expression project revealed that *HJURP* overexpression was significantly correlated with a shorter survival period of patients with head and neck cancers (dataset TCGA-HNSC; P=0.032). These data independently support the immunohistochemical data.

HJURP knockdown inhibits the growth of OC cells. To determine whether HJURP is involved in the growth of OC cells, HSC4 and Ca9-22 cells were transfected with siRNAs against *HJURP* (si-HJURP-#1 and si-HJURP-#2), along with two different control siRNAs (si-LUC and si-EGFP) with

no off-target effect on the cells as reported by our previous studies (11-37). HJURP expression was reduced in the OC cells by si-HJURP transfection as compared with the control siRNAs (Fig. 3A). Suppression of HJURP expression significantly inhibited the viability of HSC4 and Ca9-22 cells (P<0.001; Fig. 3B). Additionally, the results of the colony formation assays revealed that silencing of HJURP expression decreased the colony number of HSC4 and Ca9-22 cells, as compared to the cell transfected with the control siRNAs (Fig. 3C).

HJURP knockdown inhibits progression of the OC cell cycle. Flow cytometry was performed to clarify the functional role

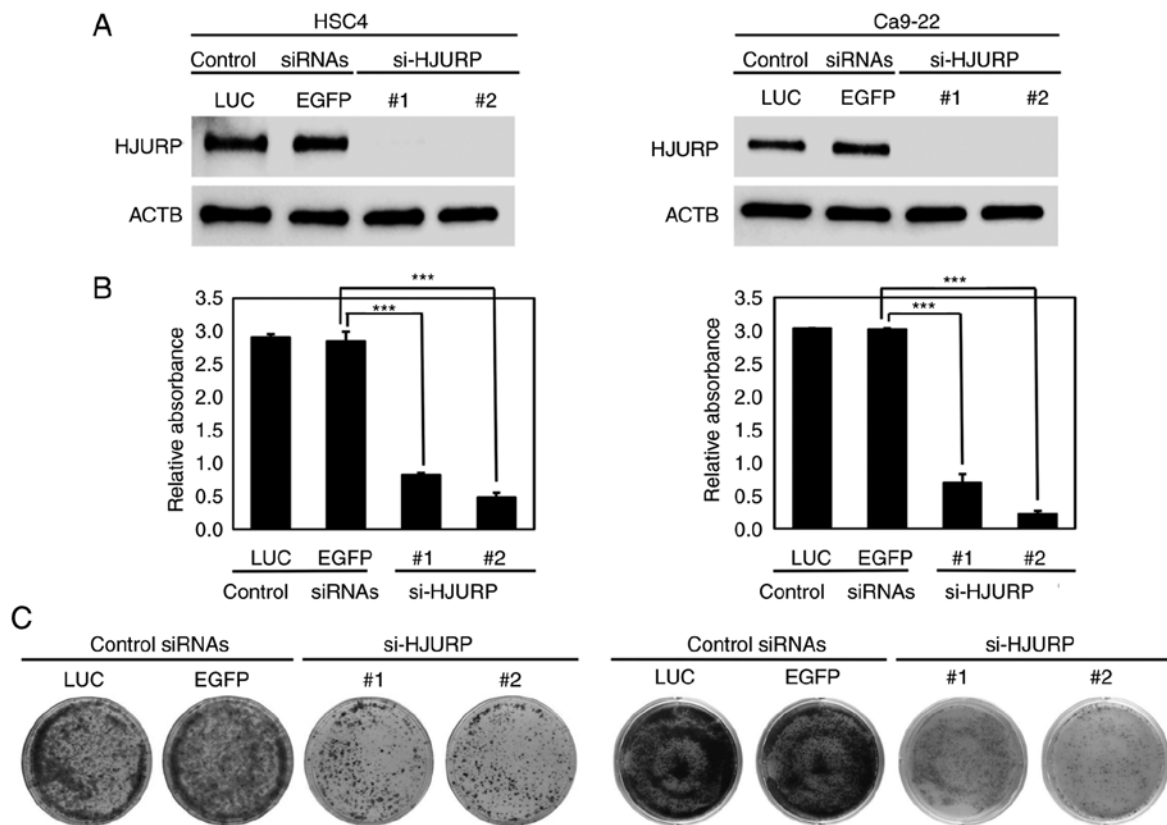


Figure 3. Inhibition of OC cell growth by HJURP knockdown. (A) Suppression of HJURP protein expression in HSC4 and Ca9-22 cells transfected with siRNAs against HJURP and control siRNAs (LUC and EGFP) as determined by western blot analysis. (B) MTT assay of HSC4 and Ca9-22 cells transfected with siRNAs against HJURP or control siRNAs. (C) Colony formation assay of HSC4 and Ca9-22 cells after transfection with siRNAs against HJURP or control siRNAs. All assays were performed in triplicate; bars, SD. *** $P < 0.001$ vs. si-control group. HJURP, Holliday junction recognition protein; OC, oral cancer.

of HJURP in the growth of OC cells (HSC4 and Ca9-22) with suppressed HJURP expression by transfection of siRNAs. The proportion of cells in the G2/M phase was significantly increased by transfection with si-HJURP as compared to control siRNA (si-LUC) (Figs. 4A and S1). In addition, cell dynamics were monitored using live imaging of HSC4 and Ca9-22 cells transfected with si-HJURP or si-LUC (Fig. 4B). Time-lapse imaging detected regular division of cells transfected with si-LUC, while very few cells transfected with si-HJURP had divided, but rather cell death was observed.

HJURP knockdown induces senescence of OC cells. Since HJURP is reported to play a role in the regulation of senescence of lung cancer cells (24), senescence-associated β -galactosidase (SA- β -Gal) staining of OC cells (HSC4 and Ca9-22) transfected with siRNAs against HJURP was performed. The results showed that HJURP knockdown increased the number of β -galactosidase-positive cells with enhanced SA- β -gal activity (Fig. 5A-C) and nuclear enlargement, which is a marker of senescence-associated heterochromatic foci (SAHF) (Fig. 5D). The expression of senescence-associated proteins in OC cells transfected with si-HJURP or si-LUC was further investigated by western blot analysis and the use of a human cytokine Ab array. After HJURP silencing, the expression levels of cyclin-dependent kinase inhibitor 1A (p21/CDKN1A) and SASP markers (IL-6, IL-8 and CCL5 in Fig. 5F and G) were increased, whereas

lamin B1 expression was reduced in the OC cells (Fig. 5E-G). These data suggest that HJURP plays a pivotal functional role in the senescence of OC cells.

Growth inhibition of OC cells by dominant-negative peptides of HJURP. HJURP is known to play roles in cell cycle progression and chromosomal dynamics by binding to CENP-A (38,39,45). In addition, the GSEA database revealed that HJURP was likely involved in various pathways, including cell cycle progression and chromosome maintenance, as well as deposition of new CENP-A-containing nucleosomes at the centromeres, which are compatible with the results of cell cycle analysis and live cell imaging of OC cells transfected with siRNAs against HJURP. The results of western blot analysis, immunocytochemistry, and the ONCOMINE database (data not shown) also confirmed the co-expression of HJURP with CENP-A in OC cells. Immunoprecipitation and subsequent western blot analysis further confirmed the binding of endogenous HJURP protein to endogenous CENP-A in OC cell lines (data not shown). Based on these data, the functional association of HJURP with CENP-A was investigated as a molecular therapeutic target.

To investigate the functional significance of inhibition of the interaction between HJURP and CENP-A on the growth of OC cells, HSC4 and Ca9-22 cells were treated with the cell permeable 11R-HJURP^{TLTY54-61} peptide, which contains a direct binding site for CENP-A or a scramble peptide. The results confirmed that 11R-HJURP^{TLTY54-61} inhibited the

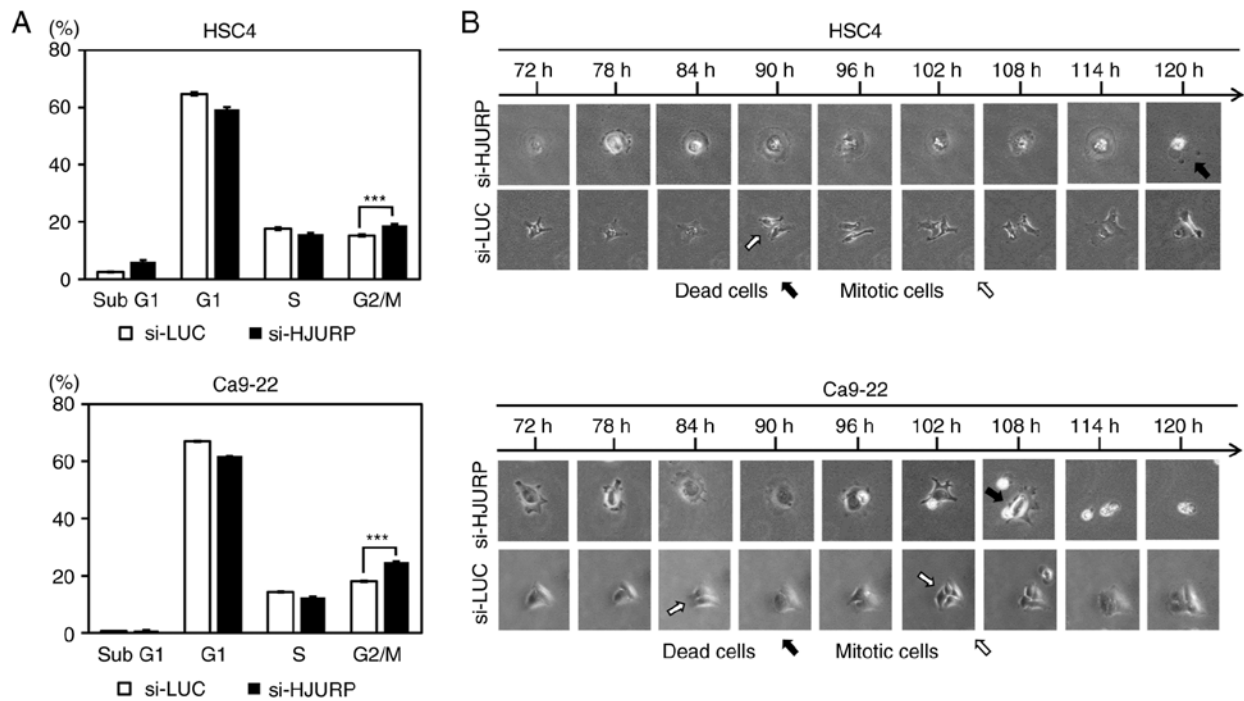


Figure 4. Inhibition of cell cycle progression and induction of cell death in OC cells by HJURP knockdown. (A) Flow cytometry of the cell cycle phase in HSC4 and Ca9-22 cells after knockdown of HJURP by siRNAs (*** $P < 0.001$ vs. si-control group). (B) Live cell imaging of HSC4 and Ca9-22 cells transfected with siRNAs against HJURP and control siRNAs. White arrow indicates mitotic cells and black arrow demonstrates dead cells. HJURP, Holliday junction recognition protein; OC, oral cancer.

interactions between HJURP and CENP-A probably through dominant-negative effects (Fig. 6A) and decreased the viability of OC cells in a dose-dependent manner (Fig. 6B). These data suggest that inhibition of the formation of functional complexes of HJURP and CENP-A is a potential therapeutic strategy.

Promotion of cell growth by enforced HJURP expression. To further confirm the cell growth promoting effect of HJURP, we transfected plasmids expressing HJURP or mock plasmids into HSC2 and SCC9 cells, which weakly expressed endogenous HJURP. Transfection of HJURP expression vector increased the viability of both cells compared with the mock plasmid as detected by MTT and colony formation assays (Fig. S2). The result demonstrated that overexpression of HJURP contributed to the enhanced cell viability of the OC cells.

Discussion

Molecular-targeted therapies have brought about a new era of treatment for various highly malignant cancers, including oral cancer (OC). Currently, monoclonal antibodies (Abs) against epidermal growth factor receptor (EGFR) (cetuximab) and immune checkpoint inhibitors (nivolumab and pembrolizumab) are available for treatment of OC. However, the efficacy is reportedly limited and various adverse events have been observed (9,10,46). Therefore, identifying new molecular targets for the development of novel therapeutics and biomarkers for precision medicine are urgently required to improve the prognosis and quality of life of OC patients. Potential target molecules with higher and frequent expression in cancer cells, but low expression in normal cells, are needed for new treatments. In the present study, Holliday junction

recognition protein (HJURP) was investigated as a potential target of the growth and survival of OC cells.

HJURP was found to be highly expressed in the majority of OC cell lines and clinical tissues, but expression was very low in normal tongue tissues and oral epithelial cells. Gene expression data demonstrated that *HJURP* expression was relatively low in normal organs (BioGPS database; <http://biogps.org/#goto=welcome>), suggesting that HJURP is a potential diagnostic and therapeutic target. Comparative genomic hybridization and genome sequencing data (<https://cancer.sanger.ac.uk/cosmic>) were referenced to assess the mechanism of *HJURP* gene aberrations in OC. The results showed that only 6 (1.4%) of 424 OC cases carried *HJURP* missense mutations. According to the cBioportal database for Cancer Genomics (<http://www.cbioportal.org/>), missense mutations, deletions, and genetic amplification of HJURP were not detected in all 40 OC cases, suggesting that HJURP overexpression might be caused by epigenetic mechanisms in oral carcinogenesis.

The tissue microarray analysis revealed that HJURP is a potential prognostic biomarker for OC, as independently validated by the ProgGene and GEPIA databases. Upregulation of HJURP in OC tissues could provide a clinical prognostic indicator that warrants intensive follow-up of patients and/or additional treatments after surgery. The present study showed that HJURP knockdown by siRNAs or inhibition of direct binding of HJURP to CENP-A by cell permeable peptides significantly inhibited the growth of OC cells probably through dysregulation of the cell cycle and/or cellular senescence. TMA analysis showed that HJURP expression is not related to tumor size. The data may reflect that HJURP knockdown is mainly targeting cell cycle regulation and/or cell survival of

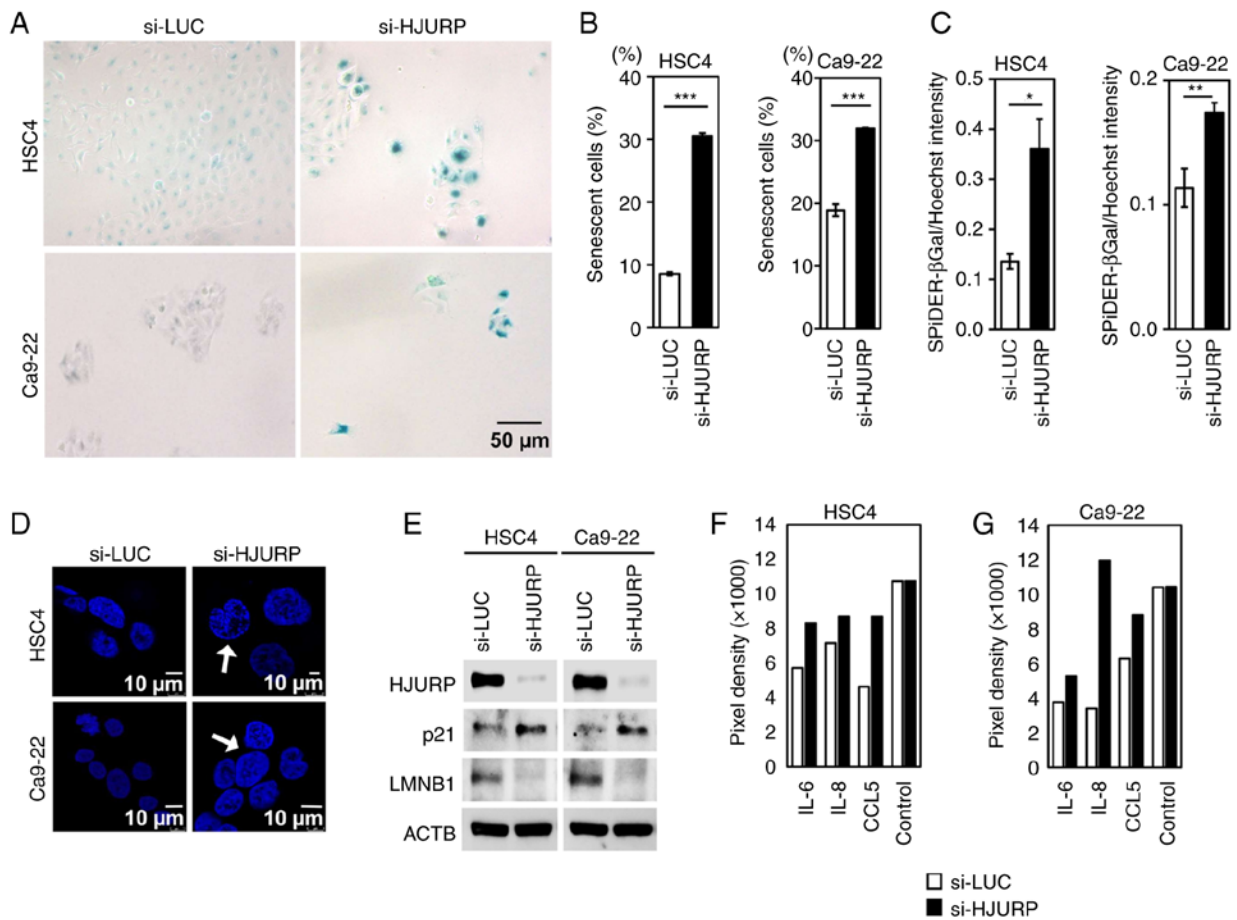


Figure 5. Induction of senescence of OC cells by knockdown of HJURP expression. (A) SA-β-Gal staining of HSC4 and Ca9-22 cells after knockdown of HJURP by siRNAs. (B) The proportions of β-galactosidase-positive HSC4 and Ca9-22 cells after HJURP knockdown detected by flow cytometry with SPiDER-βGal staining (*** $P < 0.001$ vs. si-control group). (C) Quantitation of SA-β-gal activity of HSC4 and Ca9-22 cells transfected with si-RNAs as determined with the SPiDER-βGal cellular senescence plate assay (** $P < 0.01$; * $P < 0.05$ vs. si-control group). (D) SAHF in the nuclei of HSC4 and Ca9-22 cells after knockdown of HJURP, as observed by immunocytochemistry (white arrows). (E) SASP expression in HSC4 and Ca9-22 cells after HJURP knockdown as determined by western blot analysis. (F and G) SASP expression in HSC4 and Ca9-22 cells after HJURP knockdown as determined with the human cytokine Ab array. HJURP, Holliday junction recognition protein; OC, oral cancer; SAHF, senescence-associated heterochromatic foci; SASP, senescence-associated secretory phenotype.

OC cells. Time-lapse microscopy also revealed that OC cells could not divide, and subsequently and promptly started to die. G2/M arrest and subsequent and prompt cell death after HJURP knockdown may explain the reason why the difference in the G2/M phase seems to be small, when comparing with the significant decrease in cell viability and colony numbers of OC cells. Our previous report suggested that inhibition of HJURP in lung cancer cells by siRNAs leads to the excess of chromosomal instability, G2/M arrest, as well as cellular senescence (24). Proteins such as CENP-A, MIS18A, MIS18B, and MIS18BP1 complex, and cyclin-dependent kinases interact with HJURP and regulate cancer cell cycle progression (38,39,45). Among these interacting proteins, we observed co-expression of HJURP with CENP-A protein in OC tissues, and the level of CENP-A protein was significantly reduced in HJURP-depleted OC cells, suggesting that reduction of CENP-A and/or dysregulation of unknown oncogenic pathways after HJURP knockdown might contribute to OC cell death. Therefore, targeting the interaction between HJURP and CENP-A as well as HJURP expression is likely to be one of the effective strategies for the development of new therapeutics for OC.

Transfection of HJURP expression vector is likely to increase the viability of OC cells that weakly express HJURP compared with mock plasmid; however, this needs to be confirmed by using HJURP-negative cells and conditional expression assays in a future study. Since the precise molecular mechanism underlying HJURP activation and its oncogenic role have not yet been fully elucidated, further detailed analyses of unknown oncogenic functions of HJURP as well as the mechanism about how HJURP influences the progression and senescence of OC cells through its downstream signals are warranted.

Taken together, these results revealed that HJURP is an oncoprotein that regulates OC cell growth probably by dysregulating cell cycle progression, cellular senescence, and various unknown oncogenic pathways. Since HJURP has multiple oncogenic functions and may have various binding partners as a molecular chaperon, further mechanical study of HJURP in tumorigenesis using *in vivo* models and screening of more effective approaches targeting its pathway are eagerly awaited. HJURP and its binding to CENP-A are potential molecular targets for the development of new treatments and could be useful prognostic biomarkers of OC.

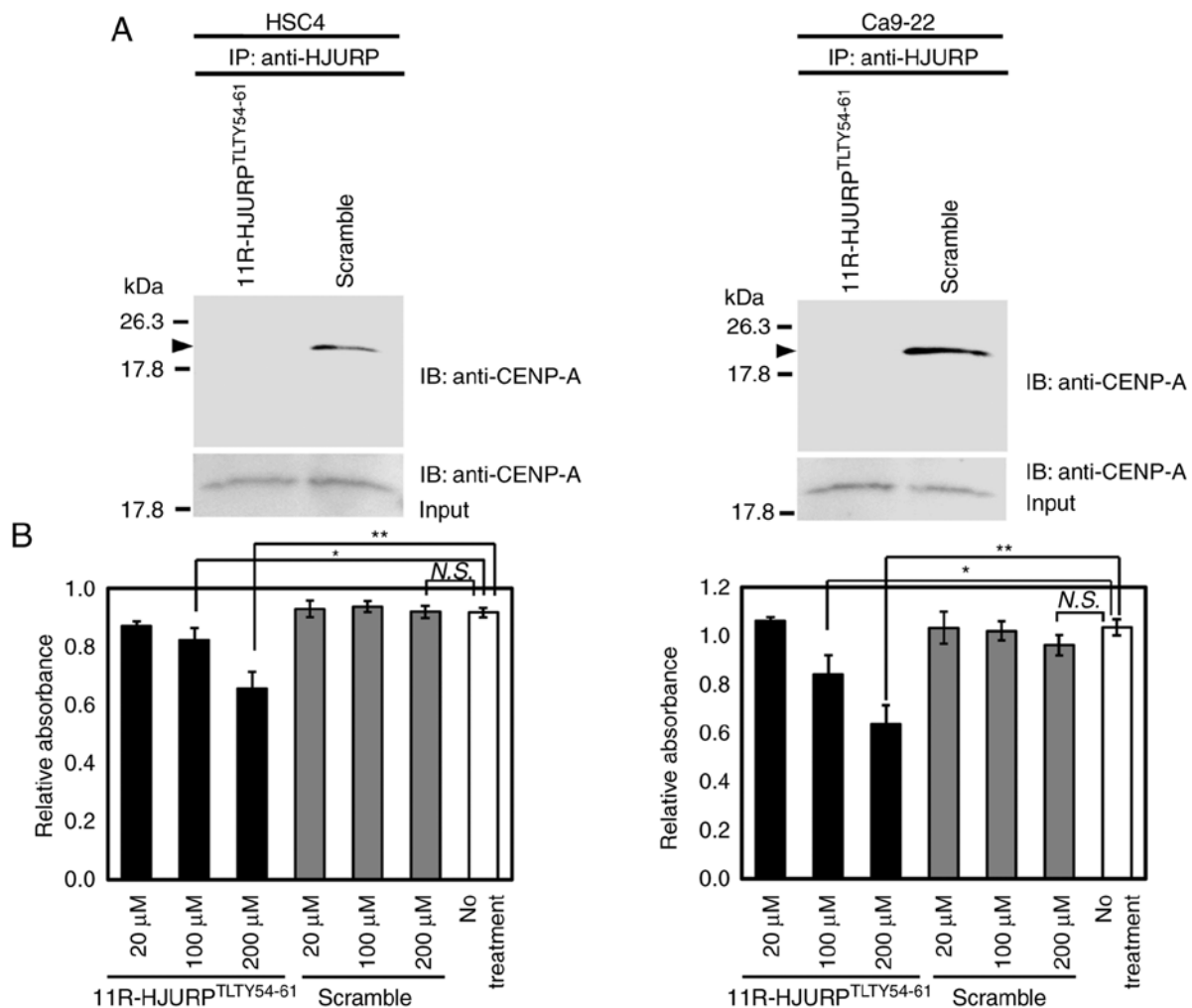


Figure 6. Inhibition of growth of OC cells by dominant-negative peptides of HJURP. (A) Reduction in the formation of complexes of endogenous HJURP and endogenous CENP-A proteins in HSC4 and Ca9-22 cells treated with 200 μ M of 11R-HJURP^{TLTY54-61} peptides or scramble peptides (top; black arrow) as determined by immunoprecipitation. (B) MTT assay showing the growth suppressive effect of 11R-HJURP^{TLTY54-61} peptides that were introduced in HSC4 and Ca9-22 cells. Bars, SD of triplicate assays. **P<0.01, *P<0.05, N.S., not significant. HJURP, Holliday junction recognition protein; CENP-A, centromere protein A; OC, oral cancer.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

BT, AT, and YD conceived the research concept and designed the study. BT, AT, and YD developed the study methodology. YY, MS, and YD acquired the data, managed the patients, and provided the facilities. BT, AT, MZ, and YD analyzed and interpreted the data (e.g., statistical analysis, biostatistics, and computational analysis) and also confirm the accuracy of the data. BT, AT, and YD wrote, reviewed, and/or revised the manuscript. BT, AT, and YD provided administrative, technical, and/or material support (i.e., reporting or organizing data, constructing databases). YD supervised the study. All authors read and approved the final version of the manuscript for publication.

Ethics approval and consent to participate

The present study and the use of clinical materials were approved by the Ethics Committees [Kumamoto University; Shiga University of Medical Science (no. G2009-163)]. It was confirmed that this study was fully ethically compliant and

the informed consent was waived due to the retrospective nature of the study and in accordance with the national ethical guidelines.

Patient consent for publication

Not applicable.

Competing interests

The authors have no competing interests to declare.

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